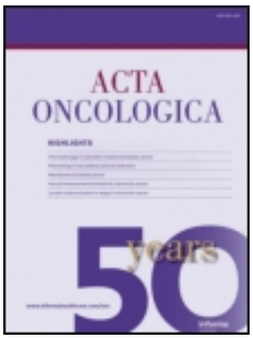

Acta Oncologica

ISSN: 0284-186X (Print) 1651-226X (Online) Journal homepage: informahealthcare.com/journals/ionc20

Lack of somatic mutations in VEGFR-2 tyrosine kinase domain in hepatocellular carcinoma

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To cite this article: Benjamin Y. S. Chuah, Wee-Lee Yeo, Jia Yi Guo, Seng-Gee Lim, Hui-Ling Yap, Boon-Cher Goh & Soo-Chin Lee (2009) Lack of somatic mutations in VEGFR-2 tyrosine kinase domain in hepatocellular carcinoma, Acta Oncologica, 48:4, 628-630, DOI: 10.1080/02841860802637781

To link to this article: https://doi.org/10.1080/02841860802637781

Published online: 08 Jul 2009.

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Lack of somatic mutations in VEGFR-2 tyrosine kinase domain in hepatocellular carcinoma

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To the Editor

Hepatocellular carcinoma (HCC), is the fifth commonest malignancy and the third leading cause of cancer mortality worldwide [1]. The majority of patients present with incurable disease with dismal

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(Received 5 November 2008; accepted 20 November 2008)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.1080/02841860802637781

prognosis. A myriad of growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and their associated receptor tyrosine kinases (RTK) result in activation of downstream pathways involved in growth, progression and metastases in HCC. One example is the RAS-RAF-mitogen-activated protein kinase/extracellular signal-related kinase kinase (MEK)/extracellular signal-related kinase (ERK) (RAS/RAF/MEK/ERK) pathway, which is controlled by RTKs activation [2]. Angiogenesis and signaling through this cascade play important roles in HCC pathogenesis, and targeting these pathways may have therapeutic implications. Indeed, sorafenib, a multi-kinase inhibitor which blocks tumor proliferation and angiogenesis by targeting VEGF receptor 2/3, PDGF receptor (PDGFR), Raf kinase, FLT-3 and c-kit, has recently been reported to improve survival in advanced HCC [3,4].

Activating tyrosine kinase (TK) mutations for various RTKs have been associated with differential responses to small molecule RTK inhibitors such as gefitinib and imatinib [5,6]. Vascular endothelial growth factor receptor-2 (VEGFR-2) is an important target for sorafenib but reliable predictors for response to their inhibition are lacking. We postulate that the presence of activating mutations in the VEGFR-2 TK domain may be associated with clinical response to small molecule RTK inhibitors targeting VEGFR such as sorafenib, in HCC, and sought to investigate this hypothesis.

Methods

We studied 66 Asian human HCC samples in accordance to institutional ethics guidelines, including 45 (68%), 3 (5%), 2 (3%) and 16 (24%) cases that were due to chronic hepatitis B infection, chronic hepatitis C virus infection, alcoholism and cryptogenic etiologies respectively. Tumor genomic DNA was extracted from fresh frozen HCC tissues using standard methods, and exons 18 to 26 of the VEGFR-2 gene, which encode the intracellular TK domain region, were amplified. All PCR reactions were carried out in 25 uL volume containing 1 ng tumor DNA in 1X PCR buffer (Promega, Madison, WI), 0.2mM deoxyribonucleoside triphosphate (Promega), 1 unit HotStarTaq DNA polymerase (Qiagen Inc., Valencia, CA) and 0.2 uM each of forward and reverse primers, using the GeneAmp PCR system 9700 (Applied Biosystems Inc., Foster City, CA). The primer sequences were as follows (5' to 3', forward and reverse):

Exon 18 (CCAAAGCAGGACATTTGGAGAGT G, CCATGCATCCTGGCATAAAGCTAC)
Exon 19 (CTGCAGATGTATTCTCCGCTTTG C, CCCTCAAACACTATCAGAGAGGCA)
Exon 20 (GGACCAGCTGATTTCTACACTCCT, TGTCCACACTCTGTAATGGGTCAG)
Exon 21 (CCCAAGTTTCAGCTCAGAGATTG C, CCTTGTAACACCCTATCACCCTGT)
Exon 22 (ATGATGAAGGCTAATGATACTGGG A, ATGGCTGACACTGGACATCTTATTT)
Exon 23-24 (GTGTGTCTCAGCAAAGCTCACA GA, CTGAGCCTGAATCTTGCACATCCT)
Exon 25 (GTCCTTCCATCCAGACTCCAAA GA, GAGCTGACGAGGTGAGATGCTAAA)
Exon 26 (TCATTGCCCAGTTAGTGTCCTGC T, AATGGCTGAGAGGATGGCATGGTA).

PCR was performed with an initial denaturation step at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C to 65°C and extension at 72°C for 1 min, followed by a final 1 min extension step at 72°C. All PCR products were purified using exonuclease and shrimp alkaline phosphatase (Amersham Biosciences, Piscataway, NJ), followed by direct sequencing with the forward or reverse primers, using the ABI PRISM Dye terminator Cycle Sequence Kit Version 3.1 (Perkin-Elmer Applied Biosystems, Foster City, CA) on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturer's recommendations. Identified mutations were confirmed by duplicating the experiment and sequencing both the sense and anti-sense strands.

Results

We did not identify any non-synonymous coding region mutations in exons 18-26 that encode for the tyrosine kinase domain of VEGFR-2 in 66 human HCC samples. One common intronic variant, *2818-37 A > G*, was identified with variant allele frequency of 0.49, while two (3%) of the HCC specimens were heterozygous for a silent variant in exon 26, *3455 G > A* (Thr > Thr).

Discussion

As VEGFR, EGFR, and PDGFR activate the RAS/RAF/MEK/ERK pathway, targeting their respective RTKs as treatment strategies is being intensively investigated in HCC. Sorafenib, which causes cancer cell apoptosis in HCC cell lines [7], improves survival in advanced HCC *in vivo* [3,4]. However, the molecular mechanism that governs VEGFR-2 activation is not fully understood, and it remains unclear how VEGFR-2 is inhibited by sorafenib. While it is known that VEGFR-2 RTKs are regulated by

tyrosine phosphorylation of activation loop tyrosine sites, it is uncertain how this regulation occurs, although phosphorylation of tyrosine in the carboxyl terminus of VEGFR-2 and leucine motif-mediated helix-helix interactions may play important roles [8]. Despite the promising clinical activity of sorafenib in HCC, we found VEGFR-2 tyrosine kinase mutations to be rare or absent in the tumor. It is possible that, unlike EGF RTK inhibitors, RTK inhibitors targeting VEGFR-2 inhibit the wild type RTK without the need of activating mutations in the TK domain. Alternative mechanisms of response include overexpression of VEGFR-2 ligands [9], presence of activating mutations in the VEGFR-2 coding regions other than the TK domain, such as the carboxyl terminus, or inhibition of alternative pathways or receptors, such as Raf kinase or PDGFR which are over-expressed and/or activated in human HCC [10,11], that are also inhibited by multi-kinase inhibitors such as sorafenib.

In conclusion, no activating mutations in the TK domain of the VEGFR-2 were identified in 66 human HCC samples. Better resolution of the crystal structure and identification of the phosphorylation sites of the VEGFR-2 may help to uncover better predictive biomarkers for VEGFR-2 inhibitors.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Evolution of glioblastoma

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To the Editor

Glioblastomas are considered fast-growing tumors, but there is little information on their growth kinetics prior to diagnosis. This is because most patients develop symptoms only when their tumors have reached a size of several centimetres [1]. We

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(Received 9 November 2008; accepted 19 November 2008)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.1080/02841860802637799